Leishmania major infections in *Phlebotomus duboscqi* fed on murine models immunized with *L. major* subcellular antigens and sandfly gut antigens

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ABSTRACT

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The ability of antibodies in bloodmeals of mice and hamsters immunized with *Leishmania major* subcellular fractions and sandfly *(Phlebotomus duboscqi)* gut antigens to inhibit development of *L. major* in its vector *P. duboscqi* was examined. Antibodies from animals immunized with either *L. major* subcellular fractions alone or sandfly gut antigen alone were not very effective in inhibiting development of *L. major* in the sandfly. When *P. duboscqi* were fed on blood from animals immunized with both parasite flagella and sandfly gut antigen, development of *L. major* was significantly inhibited ($P < 0,05$). Control sandflies fed on naive animals displayed a normal pattern of parasite development to the metacyclic stage. Electron microscopy studies showed that one of the mechanisms through which antisandfly gut antibody can cause inhibition of parasite development is by lysing sandfly gut epithelium. This study has demonstrated that it is possible to reduce transmission of leishmaniosis through immunization against both the parasite and its sandfly vector.

Keywords: Flagella antigen, *Leishmania major*, *Phlebotomus duboscqi*, sandfly gut antigen

INTRODUCTION

Prospects for the control of leishmaniosis depend to a large extent on the ability to control both the sandfly vector and the parasite. Effective control of phlebotomine sandflies has proven to be difficult because of the inaccessibility of their breeding and resting sites, and insecticide resistance (Paul, Wattal, Bhatnagar & Mathur 1978). Control of fatal visceral leishmaniosis and cutaneous leishmaniosis has proven to be as difficult as sandfly control. Experimental and clinical experience of attempted immunization against leishmaniosis with non-viable vaccines has been discouraging (Liew, Hale & Howard 1985). The only immunization strategy against leishmaniosis used so far in humans with any success has been restricted to cutaneous disease and based on convalescent immunity after controlled induction of a small lesion with low doses of virulent *Leishmania major* in an acceptable site (Gunders, Naggan & Michaeli 1972).

Studies conducted using ticks (Nyindo, Essuman & Dhadialla 1989)) and mosquitoes (Alger & Harant 1976) have shown that the host immune responses to the vector can alter the transmission of vector-borne pathogens to the host. Such immune-induced transmission blocking studies have not been reported for sandfly vectors. In their development in the sandfly, *L. major* promastigotes attach by interdigitation of the flagellum between microvilli of the cells of the abdominal midgut (Killick-Kendrick, Molyneux, Leany & Rioux 1975). In immunizing host animals with *L. major* flagellar and subcellular antigens and

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also with *Phlebotomus duboscqi* gut antigens, we hoped that the immunity generated in the host would reduce *L. major* infection rates in the sandfly by interfering with parasite development, hence reducing transmission.

MATERIALS AND METHODS

Cultivation and subcellular fractionation of *L. major* parasites

Leishmania major (strain IDUB/KE/83 = NLB-144) was isolated from a wild-caught *P. duboscqi* in Baringo District, Kenya in 1983 (Beach, Kiilu, Hendricks, Oster & Leeuwenburg 1984), and has ever since been maintained in BALB/c mice by serial subcutaneous passage. An aspirate from a footpad of an infected mouse was grown to stationary phase in NNN/Schneider's *Drosophila* medium supplemented with 20 % heat-inactivated foetal bovine serum and 250 U/mℓ penincillin, 250 µg/mℓ 5-fluorocytosine arabinoside (Kimber, Evans, Robinson & Peters 1981). Promastigotes were mass-cultivated to stationary phase concentrations of $2x10^{11}$ /mℓ. These were centrifugally washed and mixed with equal amounts of 15 % dimethyl sulfoxide in Schneider's medium and frozen at –80 °C until ready for use. Some of the primary culture parasites ($1x10^6$ promastigotes) were used to infect BALB/c mice footpads that were later used to infect sandflies.

Flagella separation and subcellular fractionation of *L. major* promastigotes

The method of Grab, Webster, Ito, Fish, Verjee & Lonsdale-Eccles (1987) was used in subcellular fractionation of *L. major* stationary phase promastigotes. Briefly, frozen parasites were thawed and centrifugally washed three times in sterile phosphate-buffered saline (PBS) at 2,500 revolutions per minute (rpm) for 30 min. The parasites ($2x10^{11}$ promastigotes) were resuspended in 35 mℓ PBS and fixed for 30 min in gas formalin. These were then disrupted by passage through a French press under a chamber pressure of 2,500 PSI into 165 mℓ SHKE buffer (a 250 mM sucrose buffer containing 25–50 mM Hepes, 25 mM KCl and 1 mM EDTA) plus 2 mℓ of 5 mg of 4 cocktail protease inhibitors (E-64, leupeptin, antipain and chymostatin) (Cambridge Research Biochemicals Ltd). The disrupted cells were centrifuged at 3,500 rpm for 10 min to obtain a pellet that contained the nuclear fraction. The supernatant was then centrifuged at 14,000 rpm to yield a pellet that contained the small and large granule. The small and large granules were resuspended in 4 mℓ SHKE and divided into two equal volumes. Into each of the 2 mℓ samples, 2 mℓ of 100 % Percoll was added and gently mixed. The 2 mℓ samples were overlaid with 2 mℓ 45 %/30 %/21 % Percoll SHKE buffer. The samples were centrifuged at 26,000 rpm for 30 min (SW-41 rotor) using a Beckman L8-80M ultracentrifuge. The entire flagella fraction was collected at the 21 % Percoll gradient. Protein estimation for the various fractions was performed using the method of Lowry, Rosebrough, Farr & Randall (1951).

Preparation of *P. duboscqi* gut for hamster immunization

Three hundred laboratory-bred, 3-d-old female *P. duboscqi* were cleaned in 2 % detergent saline to knock off all surface setae and microtrichia. These were rinsed twice in normal saline to wash off the detergent. Each sandfly was dissected in a drop of 0,15 M saline and the whole gut removed and transferred to 0,5 mℓ sterile PBS. All dissections were done on ice-cold slides to prevent protein degradation. Pooled guts were homogenized and vortex-mixed to achieve total disruption. Protein content in the crude gut antigen was determined using the method of Lowry *et al.* (1951). Gut antigen was frozen at –81 °C in batches of 20 µg of protein.

Immunization of animals using *L. major* subcellular fractions and sandfly gut antigens

Four groups of ten females 6–8 weeks old BALB/c mice each were obtained and immunized respectively with 25, 50 and 100 µg of flagella antigen in an inoculum size of 40 µℓ [20 µℓ Complete Freunds Adjuvant (CFA) 20 µℓ flagella antigen]. Mice were vaccinated subcutaneously (SC) on the rump. A similar immunization schedule was carried out using 100 µg of nuclear fraction. A control group consisting of ten mice of similar age and sex received an equal amount of PBS mixed with CFA. Mice were individually marked with picric acid for easy identification. Every week, for 2 weeks, the animals were boosted SC with the appropriate antigen which instead of CFA, was mixed in Incomplete Freunds Adjuvant (IFA), while control animals each received an inoculation comprising equal amounts of PBS and IFA. During the third week, all the mice were injected with the appropriate dose of antigen intravenously (IV) but without any adjuvant. Another group of ten mice of similar age and sex previously immunized with 50 µg to a total protein level of 200 µg of flagella antigen in CFA were immunized 2 weeks later with 7,5 µg of sandfly gut antigen, to give a total gut protein of 15 µg for this vaccine group. Four weanling Syrian golden hamsters (*Mesocrietus auratus*) weighing 140–150 g were each immunized with 20 µg crude sandfly gut protein (SGP) mixed with an equal amount of CFA. The route of immunization was intramuscular (Alger & Cabrerra 1972). At 14 and 28 d after the initial immunization, the four hamsters were boosted intraperitoneally (IP) with 20 µg of SGP, giving a total of 60 µg of SGP.

Detection of humoral immune response towards *L. major* antigen

Four weeks after the last injection, all mice were bled from the tail into heparinized 100 μℓ capillary tubes. The blood samples were then transferred onto Whatman filter papers. The serum on the filter papers was eluted with PBS-T_{20} and that emanating from each group of mice was grouped into a sample on which ELISA was carried out. Initial checker-board ELISA results showed that a serum dilution of 1:50 and a coating antigen concentration of 50 μg gave optimum readings. ELISA for detecting *L. major* flagella specific IgG antibodies was performed as previously described by Voller, Bartlett & Bidwell (1976). Briefly, U-well polyvinyl chloride microtiter plates (Dynatech Laboratories, USA) were coated overnight at 4 °C with 100 μℓ of 50 μg of *L. major* flagella antigen. The plates were washed three times, blocked with 5 % bovine serum albumin in phosphate buffer containing 0,05 % PBS-T_{20} and, 200 μℓ of mouse serum added at a dilution of 1:50 in PBST. Sera were tested in duplicate. Plates were incubated for one hour at 4 °C and at room temperature respectively and washed. Rabbit antimouse IgG (whole molecule) peroxidase conjugate (Sigma(R)) was added at a recommended working dilution of 1:2000 and incubated for 2 h at room temperature. The plates were thereafter washed and then 100 μℓ of 0,4 mg/mℓ substrate [o-phenylenediamine o-Diminobenzene dihydrochloride] (Sigma(R)) and 30 % hydrogen peroxide (Sigma(R)) added. These were then incubated for one hour in the dark at room temperature. The reaction was stopped by adding 25 μℓ of 1 N HCl (BDH, UK), and the optical density read using a Titertek(R) ELISA reader using a 492 nm filter. The hamsters were bled by snipping the tail 2 weeks after the second boost and their blood was used for detecting *P. duboscqi* gut specific IgG antibodies using a sandwich ELISA.

Sandfly feeding on mice immunized with *L. major* subcellular antigens and a hamster immunized with sandfly gut antigens

The effect of specific antibodies and probably other effector cells against *L. major* subcellular fractions, sandfly gut antigen and a combination of flagellar and gut antigen ("cocktail vaccine") on parasite development in sandflies was assessed. An average of forty two sandflies per experiment was utilized. Briefly, the sandflies were separately fed on a footpad lesion of two *L. major*-infected BALB/c mice and after they had ingested a small amount of blood, they were transferred (interrupted feeding) to the footpads of mice previously inoculated with flagella antigen at a dosage rate of 25 μg (total protein [TP] = 100 μg) or 100 μg (TP = 400 μg); nuclear fraction at a rate of 100 μg (TP = 400 μg); "cocktail vaccine" (flagella, TP = 200 μg; gut, TP = 60 μg); or PBS, or to the footpad of a hamster immunized with 60 μg *P. dubosqi* gut antigen. After these sandflies had ingested a small amount of blood from inoculated animals, they were again interrupted and transferred to the lesion. This process was repeated until all the sandflies were fully engorged, to ensure that they had taken almost the same amounts of blood from both the infected mouse and the immunized animals. The groups of engorged sandflies were kept separately in the insectary at 25 °C and a relative humidity of 90 % for 6 d. During this time, they were given sugar syrup as an energy supplement. On the seventh day, they were dissected using the method of Johnson, McConnell & Hertig (1963) and examined for the presence of fully formed and developmental stages of parasites and their positions in the gut.

Pathological studies on the effect of anti-sandfly gut antibodies on the guts of *P. duboscqi*

To determine whether there was any damage in the gut of the sandflies caused by antibodies against gut antigen, another group of sandflies comprising 30 individuals were fed on the hamster immunized with 60 μg gut protein. On each of days 2, 3 and 6 post-feeding, 5 of the flies were anaesthetized and washed in 2 % detergent to remove setae. Five unfed naive *P. duboscqi* were used as controls. Guts were dissected and pre-fixed overnight in 2,5 % glutaraldehyde (Mecrk(R)) in 0,1 M cacodylate buffer (pH 7,2–7,4) at 4 °C. Tissues were then washed in 0,1 M cacodylate and then post-fixed in 0,1 % osmium tetroxide (TAAB(R)) in 1,0 M cacodylate buffer and then serially dehydrated in increasing concentrations of analytical grade ethanol starting with 30 %, followed by 50 %, 70 %, 80 % and, lastly, 96 % ethanol. They were placed in each solution for a period of 15 min. Thereafter, the specimens were placed in a 1:1 mixture of absolute ethanol/ propylene oxide/epon overnight in the hood to enable them to be gradually infiltrated by it, and finally they were immersed in 100 % epon 812 resin mixture in the hood. Embedding was done in an epon resin mixture which was allowed to polymerize at 60 °C in an oven for 48 h. Transverse and longitudinal sections were cut using a Reichert-ultramicrotome. These were double stained with lead citrate and urinal acetate and viewed in a Joel 100S transmission electron microscope (EM).

RESULTS

ELISA results: Humoral response to flagella antigen

Sera from mice immunized with *L. major* nuclear antigen had significantly higher absorbance value than mice inoculated with flagella antigens and control PBS mice, indicating a successful immunization.

However, absorbance value of sera from the three different flagella doses (25, 50, 100 µg) used to immunize the animals were not significantly different from each other (Table 1). The nuclear fraction had the highest OD of 0,70, an indication that it elicited a good humoral response compared to the flagella antigen.

Leishmania major development in *P. duboscqi* fed on mice and hamsters

Examination of sandflies fed on immunized animals revealed that inhibition of parasite development was most pronounced in sandflies fed on mice immunized with the "cocktail vaccine", where only 2/19 (10,53%) sandflies were infected and 17/19 (89,50%) were not infected. Results of infections in sandflies fed on mice immunized with the various parasite and sandfly antigens are summarized in Table 2. A chi-square (χ^2) analysis of the number of sandflies with *L. major* infection showed that inhibition of parasite development due to bloodmeals from mice immunized with the "cocktail vaccine" was significantly higher than that in sandflies fed on the control mice inoculated with PBS ($\chi^2 = 7,09$, degrees of freedom [df] = 1, $P < 0,01$). Inhibition of parasite development by bloodmeals from animals immunized with the "cocktail vaccine" was significantly higher ($P < 0,01$) than that caused by bloodmeals from mice immunized with flagella antigens (25 and 100 µg), nuclear fraction, and bloodmeal from a hamster immunized with sandfly gut antigen.

Bloodmeal from mice immunized with 25 and 100 µg of flagella antigen inhibited development of parasites in 75,76% and 72,73% of the engorged sandflies respectively. These levels of inhibition caused by bloodmeals from mice immunized with two flagella antigens were not significantly different ($\chi^2 = 0,06$, df = 1, $P < 0,05$). Bloodmeals from nuclear fraction-immunized mice and a hamster immunized with gut antigen alone inhibited *L. major* development in 59,1 % and 30,4 % of engorged sandflies respectively.

The different developmental stages of *L. major* that were seen in dissected sandflies are shown in Table 3. The forms and positions of parasites in sandfly guts were examined in wet preparations soon after dissection and in Giemsa-stained smears. *Phlebotomus duboscqi* fed on mice immunized with the "cocktail vaccine" and flagella antigens had mainly nectomonads and haptomonads in the thoracic and abdominal midgut. Very few of these sandflies had metacylic forms. Sandflies fed on mice and hamsters immunized with either gut, nuclear fraction or con-

TABLE 1 Grouped mice antibody response to *L. major* subcellular antigens and from a hamster immunized with *P. duboscqi* gut antigen

Immunizing antigen	Protein amount (µg)	OD
Flagella	100	0,46
Flagella	50	0,41
Flagella	25	0,42
Nuclear fraction	50	0,70
PBS	0	0,06
Gut	60	0,02

TABLE 2 Infection rates in *P. duboscqi* fed on mice and hamsters immunized with *L. major* subcellular antigens and sandfly guts, respectively

	Blood fed *P. duboscqi*			Unfed *P. duboscqi*		
	Total fed	No. with parasites	No. without parasites	Total unfed	No. with parasites	No. without parasites
Flagella (25 µg)	(63,46)[a] 33/52	(24,24) 8/33	(75,76) 25/33	(36,54) 19/52	(15,79) 3/19	(84,21) 16/19
Flagella (100 µg)	(56,41) 22/39	(27,27) 6/22	(72,73) 16/22	(43,59) 17/39	(17,65) 3/17	(82,35) 14/17
Nuclear fraction	(53,66) 22/41	(40,91) 9/22	(56,09) 13/22	(46,34) 19/41	(15,79) 3/19	(84,21) 16/19
Gut (60 µg)	(71,88) 23/32	(69,57) 16/23	(30,43) 7/23	(28,13) 9/32	(11,11) 1/9	(88,88) 8/9
"Cocktail"	(59,37) 19/32	(10,53) 2/19	(89,47) 17/19	(40,63) 13/32	(23,08) 3/13	(76,92) 10/13
PBS	(59,26) 32/54	(46,88) 15/32	(53,13) 17/32	(40,74) 22/54	(9,09) 2/22	(90,99) 20/22

[a] Numbers in parentheses are percentages

TABLE 3 Parasite forms seen in guts of *P. duboscqi* fed on mice immunized with *L. major* subcellular fractions and a hamster immunized with sandfly-gut antigen

Immunizing antigen	Parasites position and forms seen		Commonest form seen
	Midgut	Stomodeal valve	
Flagella (25 µg)	Nectomonads Haptomonads	Few Metacyclics	Nectomonads Haptomonads
Flagella (100 µg)	Nectomonads Nectomonads	Few Haptomonads Few Metacyclics	Haptomonads
Nuclear fraction	Nectomonads Haptomonads	Haptomonads Metacyclics	Haptomonads Metacyclics
Gut (60 µg)	Haptomonads Nectomonads	Haptomonads	Haptomonads Metacyclics
"Cocktail"	Few Haptomonads and nectomonads	Few Haptomonads	Nectomonads
PBS	Nectomonads Haptomonads	Haptomonads Metacyclics	Metacyclics

trol PBS supported full development of *L. major* leading to development of metacyclic promastigotes. Transverse and longitudinal sections of gut of the sandflies fed on the hamsters immunized with 60 µg gut protein revealed degeneration of gut epithelium on days 2 and 3 post-feeding when examined under the EM. On days 5 and 6 after feeding on the immunized blood, there was regeneration of the epithelium. In the gut of the unfed control sandflies, no deviations from normal were observed.

DISCUSSION

Host antibodies that interfere with aspects of mosquito physiology have been shown to hinder the development of certain arboviruses in the vector, hence deleteriously affecting successful transmission of parasites (Ramasamy, Sands, Kay, Fanning, Lawrence & Ramasamy 1990). Antibodies against midgut components of *Aedes aegypti*, when ingested with an infected bloodmeal, have been shown to reduce the susceptibility of the mosquito to infection with arboviruses (Ramasamy *et al.* 1990). Similarly, it has been demonstrated that antibodies generated by mice immunized with *Anopheles farauti* midgut antigens significantly reduce the number of *Plasmodium berghei* oocysts developing in the mosquito (Ramasamy & Ramasamy 1990). Our observations in this study have shown that antibodies against *P. duboscqi* gut and *L. major* flagella are able to significantly inhibit development of infective promastigotes, when an infected bloodmeal is ingested with blood from immunized animals.

The inhibitory effect of antibodies against *L. major* flagella or sandfly gut antigen when the two are ingested separately was low compared to when the two were ingested together in a single bloodmeal. This observation suggests that the anti-*L. major* flagella antibodies and *P. duboscqi* gut antibodies may have a synergystic action. In a previous study, Mbati, Anjili, Lugalia, Mwanyumba, Tonui, Robert & Githure (1995) demonstrated that BALB/c mice immunized with a cocktail vaccine composed of a mixture of *L. major* flagella (50 µg) and *P. duboscqi* gut (60 µg) significantly protected mice against a challenge infection with virulent *L. major*. Flagella or gut antigen when used alone did not confer any protection. In another related study, Ingonga, Mbati, Anjili, Mutani, Wishitemi, Robert & Githure (1996) showed that *P. duboscqi* fed on hamsters immunized with *P. duboscqi* gut antigen exhibited a significant increase in mortality and a decrease in fecundity. Taken together, these observations, and the finding that antibodies against *P. duboscqi* gut cause lysis of midgut epithelium suggest that it is possible to limit transmission of *L. major* by immunizing a susceptible host against the parasite and the vector simultaneously.

The actual mechanism of action of both flagella and gut antibodies could not fully be determined in this study. It is possible that antibodies against flagella act by opsonizing the flagellum and rendering it incapable of interdigitating with sandfly gut microvilli. This kind of action may also prevent flagella insertion and hence parasite development. The antibodies against sandfly gut may act by lysing gut epithelium

thus disrupting microvilli continuity which is important for flagella insertion during parasite maturation. From our results it appears that the lethal effects of anti-sandfly gut antibodies (gut lysis) is high during the first 3 d after feeding. During the fifth and sixth days after the immune bloodmeal, there is regeneration of epithelium as most of the bloodmeal containing the antibody is digested. These observations are consistent with those of Ingonga *et al.* (1996) who found that the highest mortality rate in *P. duboscqi* fed on hamsters immunized with sandfly gut antigen occurs within 72 h after the immune bloodmeal. The mortality was found to be dependent on the dose of the immunizing antigen, and was seen to decrease with time until day 6 when it was nil.

In this study, in most of the sandflies that were dissected, the commonest parasite forms that were seen were nectomonads and haptomonads. It is possible that since parasites used in preparing the *L. major* antigen were metacyclics, the antibodies were mainly directed at the metacyclic flagellum. This is because it has been shown that protection due to immunization against *L. donovani* infection is determined by the growth phase of the parasite at the time of antigen preparation, with the stationary phase parasites offering the best protection (Jarecki-Black, James, Kirshtein, Kirshtein & Glassman 1986). The low level of inhibition of *L. major* development in the sandfly elicited by the nuclear fraction compared to the "cocktail vaccine" may mean that protection is mainly conferred by antibodies against extrinsic parasite epitopes. This study has demonstrated that it is possible to use a vector/parasite based transmission-blocking vaccine to limit transmission of leishmaniosis. Such a transmission blocking vaccine would be practicable as part of an integrated control strategy against anthropogenetic leishmaniosis, such as kala azar in India and Kenya.

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